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Loss-of-Function Mutations in the Filaggrin Gene Lead to Reduced Level of Natural Moisturizing Factor in the Stratum Corneum

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TO THE EDITOR

Filaggrin is a key protein required for the formation of the stratum corneum (SC) barrier. Filaggrin is also essential for SC hydration, as it acts as a source of hygroscopic amino acids and their derivatives, known as natural moisturizing factor (NMF).

The human gene encoding filaggrin (*FLG*) is highly polymorphic and to date, 15 null mutations have been detected of which four (R501X, 2282del4, R2447X, and S3247X) are prevalent at varying frequencies in the white European population (Sandilands *et al.*, 2007). Homozygous or compound heterozygous *FLG* mutations underlie the common skin-keratinizing disorder ichthyosis vulgaris, and have been shown to be a major genetic predisposing factor for atopic dermatitis (AD) (Sandilands *et al.*, 2006). Diminished filaggrin expression has been demonstrated in both ichthyosis vulgaris and AD skin (Seguchi *et al.*, 1996; Sugiura *et al.*, 2005; Smith *et al.*, 2006).

As filaggrin is the precursor protein for the amino-acid-derived components of the NMF, we hypothesized that carriers of *FLG*-null mutations have reduced level of NMF in the SC.

To measure NMF in the SC of the palm (thenar eminence) and forearm skin, we used confocal Raman microspectroscopy (3510 Skin Composition Analyzer; River Diagnostics, Rotterdam, The Netherlands). The principles of this method and the procedure have extensively been described elsewhere (Caspers *et al.*, 2001, 2003). The reference spectrum of NMF was constructed from a superposition of the spectra of pyrrolidone-5-carboxylic acid, ornithine, serine, proline, glycine, histidine, and alanine.

In addition to NMF, skin barrier function as measured by transepidermal water loss was assessed on the volar forearm (Tewameter 210; Courage and Khazaka Electronic GmbH, Cologne, Germany).

One hundred and forty-nine volunteers recruited by public advertisement, as well as 10 AD patients, were screened for four *FLG* mutations (R501X, 2282del4 R2447X, and S3247X). All subjects filled in a questionnaire on the history of skin diseases and allergies, and the Erlangen atopy questionnaire that also included a question on skin dryness. Signs of active disease (erythema, crusting, weeping, and lichenification) were assessed by a dermatologist. Having visible skin changes on the forearm was the exclusion criterion. Written informed consent was obtained from all subjects. The experimental protocol followed the Declaration of Helsinki Principles and was approved by the Ethical Committee of the Academic Medical Centre. Genomic DNA was extracted from buccal swab samples (Puregene® DNA isolation kit; Gentra Systems, Minneapolis, MN). Polymorphisms were genotyped as reported previously (Sandilands *et al.*, 2007). To compare data from two groups, we used two-tailed Student's *t*-test for unpaired samples.

Sixteen carriers (12 female) of an *FLG* mutation and 23 individuals (15 female) wild type with respect to these mutations were included in the study. Of the 16 carriers, five were heterozygous for R501X, eight were heterozygous for 2282del4, and one was heterozygous for R2447X. One individual was homozygous for

Abbreviations: AD, atopic dermatitis; FLG, *human filaggrin-encoding gene; NMF, natural moisturizing factor; SC, stratum corneum*

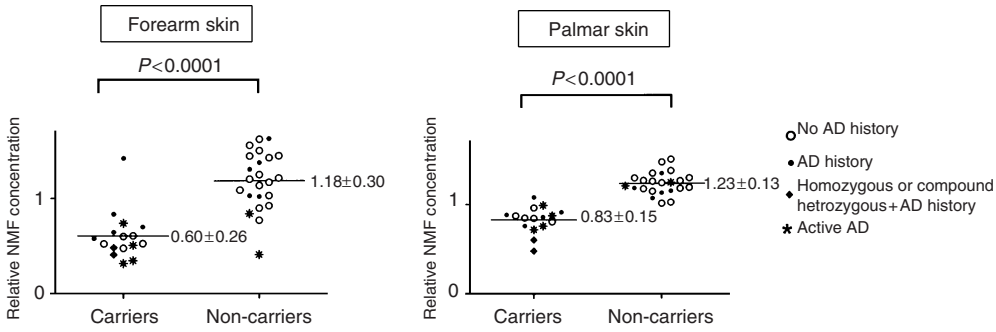

Figure 1. The individual and average (±SD) values of relative NMF concentration in the SC of the forearm and palm of carriers and non-carriers of a *FLG* loss-of-function mutation. Relative NMF concentration is expressed as the NMF/keratin ratio in arbitrary units.

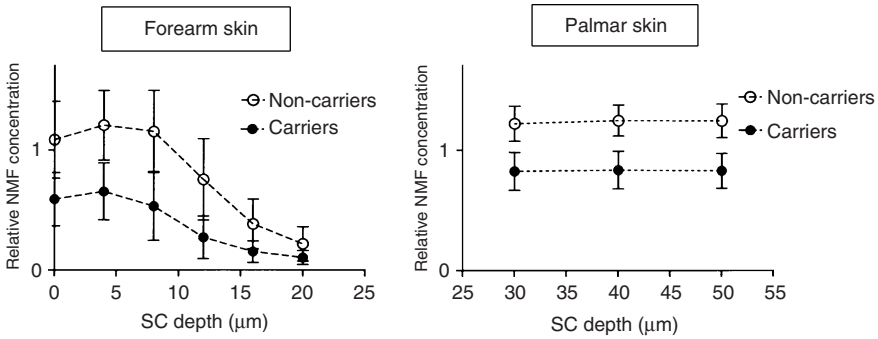

Figure 2. Relative NMF concentration (mean ± SD) measured at different SC depths on the forearm and palm of carriers and non-carriers of *FLG* loss-of-function mutations. Relative NMF concentration is expressed as the NMF/keratin ratio in arbitrary units.

2282del4 and one individual was compound heterozygous for R501X and 2282del4. The mean age of the carrier group was 33 years (range 20–55 years), which was comparable with that of non-carriers (mean 30, range 20–61 years). The prevalence of AD history and active AD was higher in carriers of an *FLG* mutation (Figure 1). Further, all carriers and 11 non-carriers reported having a dry skin.

In this study, we have demonstrated that individuals who are carriers of *FLG*-null mutations have significantly reduced levels of NMF in the SC on both skin locations and at all SC depths (Figures 1 and 2). Palmar skin showed less interindividual differences in NMF levels as compared with forearm skin (Figure 1). *FLG* carriers who had signs of active disease ($n=4$) did not have NMF values different from those in individuals without active AD (both skin locations $P>0.1$), although we acknowledge that the size of groups are small. Also a history of AD did not result in different NMF levels within both groups ($P>0.1$). Furthermore, *FLG* carriers with a history of AD had significantly lower NMF level than non-carriers with a history of AD ($P<0.0001$). Two individuals who were homozygous and compound heterozygous for investigated *FLG* mutations showed lower NMF levels than the group averages (Figure 1).

Although clear difference was found in NMF content between carriers and non-carriers of *FLG* mutations, it has to be realized that NMF content is dependent on several factors and not only on the *FLG* genotype (Rawlings and Matts, 2005). The breakdown of filaggrin is under control of proteolytic enzymes and is influenced by the hydration gradient across the skin that is dependent on the external humidity and the transepidermal water loss. The latter factor is influenced by the quality of the skin barrier, primarily by the composition and structure of the lipid bilayers. There are also other possible genetic modifiers of the effect of *FLG* mutations, which are plausibly involved in controlling filaggrin processing, including *SPINK5* and *SCCE* (Sandilands *et al.*, 2007). In addition, a heterozygote for a loss-of-function mutation might carry an expanded exon 3 on the other allele, lessening the overall effect of the mutation (Smith *et al.*, 2006). Furthermore, it has recently been reported that filaggrin skin expression could be modulated by the atopic inflammatory response mediated by cytokines IL-4 and IL-13 (Howell *et al.*, 2007).

In addition to reduced levels of NMF, carriers of *FLG* mutations showed a higher transepidermal water loss as compared with non-carriers (10.3 ± 2.7 and $8.3 \pm 2.2\,\mathrm{g\,hm^{-2}}$, respectively; $P=0.01$). The literature data on transepidermal water loss in AD patients is contradictory, and this inconsistency might, at least partly, be explained by different phenotypes of AD patients. Given that approximately 50% of moderate-to-severe AD patients may be carriers of an *FLG*-null mutation, it has been speculated that *FLG* status may define a subtype of barrier-related AD. Since polymorphisms in the *FLG* gene lead to reduced amount of filaggrin and

consequently to a decreased level of NMF, measurement of NMF could improve classification of AD phenotypes. Identification of this measurable physical parameter as a marker of *FLG* status could enable more targeted prevention of AD in susceptible individuals. Measurement of the NMF phenotype in particular by *in vivo* Raman spectroscopy, is much less demanding then genotyping. This initial study shows some highly promising results. Sensitivity and specificity of this method will be further investigated in larger, well-defined study groups to explore its potential usefulness in clinical practice.

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene. The other authors state no conflict of interest.

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Depletion of Human Peripheral Blood Lymphocytes in CD25 + Cells Allows for the Sensitive *In Vitro* Screening of Contact Allergens

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TO THE EDITOR

Allergic contact dermatitis (ACD) is a frequent T-cell-mediated inflammatory skin disease in response to skin exposure to non-protein chemicals, called haptens. Development of ACD proceeds in two phases (Saint-Mezard *et al.*, 2004). The sensitization (afferent) phase, induced by the migration of hapten-loaded skin dendritic cells (DCs) to draining lymph nodes, leads to the priming of hapten-specific effector T cells. The elicitation (efferent) phase, generated on skin re-exposure to the same hapten, induces the recruitment of T cells into the skin resulting in ACD through IFN-γ production and cytotoxicity toward keratinocytes.

Allergic contact dermatitis is a major public health concern in industrialized countries, and there is an urgent need for tests allowing for detection of potentially allergenic compounds manufactured by industries (Diepgen and Kanerva, 2006; Williams *et al.*, 2006). Predictive testing for the sensitizing properties of haptens has been traditionally carried out in guinea pigs and mice (Gerberick *et al.*, 2007; Maurer, 2007). More recently, alternative *in vitro* methods have been developed to test the ability of haptens to prime naive peripheral blood T cells. These assays, referred to as primary sensitization assays, consist of an autologous mixed DC–lymphocyte reaction (MDLR) using hapten-loaded DCs as antigen-presenting cells and normal human peripheral blood leucocytes (PBLs) as responding cells. *In vitro* priming of hapten-specific T cells was usually assessed by cell proliferation

Abbreviations: ACD, allergic contact dermatitis; DC, dendritic cell; MDLR, mixed DC–lymphocyte reaction; PBL, peripheral blood lymphocyte